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Facile bioconversion of vegetable food waste into valuable organic acids and green fuels using synthetic microbial consortium

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Abstract−The production of various organic acids from vegetable waste *via* a facile and cost-effective method utilizing characterized synthetic microbial consortia is described in this study. Five bacterial species with the ability to produce organic acids from vegetable waste biomass were isolated and identified as *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus megaterium*, *Pseudomonas florescence* and *Escherichia coli*. Using these cultures, mixed acid fermentation was developed and was efficient in producing various organic acids. The total organic acids accumulated using optimized fermentation conditions was found to be 72.44±3.43 g L^{-1}. The acetic acid was produced as major acid accumulated up to 25.27±1.26 g L^{-1}, followed by lactic acid 19.11±1.73 g L^{-1}. Efforts were also put forth to check the ability to produce methane by the anaerobic digestion process. Up to 14.97 mL g^{-1} biomass methane was produced during the anaerobic digestion process. The technology developed in this study is a carbon-neutral process for managing vegetable food waste with economic benefit. The developed technology will have great economic potential and add value to vegetable food waste management.

Keywords: Carbon Footprint, Landfill Disposal, Organic Acids, Synthetic Microbial Consortia, Vegetable Waste Biomass

INTRODUCTION

In recent years the keen interest of industries shifted from depending on traditional hydrocarbon-based carbon building blocks towards green synthesis via microbial fermentation. Organic acids come under the carboxylate platform and act as biobased chemical building blocks that can help in the green synthesis of many valuable chemicals [1,2]. The library of organic acids such as acetic acid, oxalic acid, lactic acid, propionic acid, butyric acid, fumaric acid, malic acid, succinic acid, itaconic acid, citric acid, glucaric acid, and gluconic acid, can be derived from microbial fermentation and acts as chemical raw material. These acids range from C_2 to C_6 and are presently produced at the commercial level by microbial fermentation, occupying up to 5-10% of the total industrial production scale [1]. In recent years, higher chain diacids also have been reported *via* whole-cell biocatalysis utilizing vegetable oils with the help of recombinant strains [2,3]. These organic acids have applications as feedstocks in the synthesis of chemicals whose utilities start from food additives and act as raw material to produce polymers, synthetic intermediates, pharmaceutical agents, metal chelator, nylon, polyester production, therapeutics, fragrance/aroma ingredients, and in the synthesis of biodegradable polymers, complexing agents and also used as green solvents [1,4]. However, this adaptation is not in the desired acceleration to attain success at the commercial level. This hindrance is due to the cost incurred for the production of organic acids via microbial fermentation since biorefinery relies on costly culture media and the defined nutrients. Hence, researchers are looking to utilize low cost or waste biomass to produce organic acids; thus the production cost comes down and can attain commercial feasibility.

Urbanization is accelerating in larger cities, leading to the increased discharge of vegetable/food waste from the domestic and food industries. The vegetable/food waste discharge is presently a major urban issue because the protocols used in waste management elicit carbon emissions. Most of the nation's local governance primarily manages waste discharge *via* the landfilling method. Landfilling comes under the non-green disposal method as the wet solid waste, which includes the vegetables, food, kitchen waste generated by the domestic/commercial/food market/food processing industry, is rich in carbon and nitrogen sources along with the macro and micronutrients. Hence, is very much suitable for microbial growth [5,6]. In conventional landfilling disposal, the wet food waste upon the disproportionate microbial activity will result in methane and other greenhouse gases released into the environment. Hence, causing global carbon footprint accumulation [7]. According to the National Food and Agriculture Organization, up to 8% of global carbon footprint emission is due to improper vegetable/food waste management. Moreover, it is also responsible for local pollution, groundwater perturbance, and the release of toxic greenhouse gases through anonymous decomposing activities by the various microbiome. Hence, many countries with a high population density, e.g., the Republic of Korea, have banned landfilling disposal methods for vegetative food waste and/or domestic kitchen waste [8]. Thus, environmental research has shifted towards utilizing this waste to generate biochemical building blocks like organic acids. Organic acids can act as raw materials for the green synthe-

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sis of chemicals and fuels *via* proportionate microbial fermentation technology. The production of organic acids is becoming a priority from an economic perspective because they have versatile applications as organic building blocks for the green synthesis of valuable chemicals. Thus, researchers are interested in creating prospective technology with majorly two agendas: implementing green waste management practices which can bypass the economic input, and saving the space for landfilling disposal and creating sustainable economic addition to vegetative waste management.

Shifting from the dependence of hydrocarbon raw materials for synthesizing various chemicals to green synthesis methods utilizing renewable biological building blocks (organic acids) is strongly recommended to industries. From the couple of decades, global industries have been getting sensitized towards green synthsis of various chemicals. Besides, the national-international bodies are coming into action for the future goal of environmental sustainability, forcing many industries to adopt biological-based green feedstocks, replacing the traditional process, and making each industrial process green and carbon neutral. Organic acids can act as feedstock for the green synthesis of many industrially important chemicals which are presently generated through traditional non-green methods. Green synthesis of chemicals from the feedstocks generated through microbial cell factories can have a dual advantage. Firstly, it produces economically important chemical building blocks; secondly, it helps to reduce the carbon emissions released by vegetable food waste during the rotting of landfilled disposal. Also, utilizing hydrocarbon feedstocks in turn adds extra carbon footprint to the environment. Hence, utilization of energy-rich vegetable food waste to generate organic acids would be a good solution to cut down the cost of the product and direct/indirect carbon footprint release to the environment. This technology will reduce the end-product cost several-fold and circumvent the problem of waste disposal.

To date, many studies have been reported for producing organic acids by various microbial fermentation processes; however, most of these studies are based on selective single pure culture fermentation or mixed culture microbial consortia generated *via* selective adaptation. These utilize a defined nutrient medium to attain a high titer of the target product in the bioreactor. In the case of a prior condition, the use of single pure culture will result in a single organic acid; and the latter, using mixed microbial consortia has the problem of inconsistency due to variations in representative microbial population during fermentation. Thus, these technologies could not withstand commercial success. Hence in this study, the effort was put forward to generate a facile and cost-effective method of conducting mixed culture fermentation to produce multiple organic acids. This is demonstrated by utilizing characterized microbial consortia taking vegetable food waste as a nutrient supplement. The process developed in this study could produce 72.44±3.43 g L^{-1} of total organic acids and methane up to 14.97 mL g^{-1} biomass.

MATERIAL AND METHODS

1. Biomass, Media Components and Inoculum Preparation

Vegetable waste collected from the local market Daganiya Chowk and Gol Chowk, Raipur, Chhattisgarh, India [21.2361N, 81.62997, 21.25000, 81.62997] was used as the organic biomass for the fermentation culture medium. The freshly collected vegetable waste was washed with regular tap water, then blended with a domestic electric mixer to prepare the thick vegetable slurry and used in the experiments. Minimal buffer medium containing the 10 mM phosphate buffer containing the following salt components in one liter: 0.034 g $FeSO_4$, 1.0 g CH_3COONa, 1.23 g $MgSO_4{\cdot}7H_2O$, 0.034 g $MnSO_4{\cdot}H_2O$, 0.65 g $2HPO_4{\cdot}3H_2O$, 0.5 g KH_2PO_4. For growing seed culture, 10 g of yeast extract and 2 g of tryptone were added to the above components. The slurry was diluted as desired according to the experimental design described in the results section. The modifications if any were done according to the experiments and were detailed in the respective results section.

2. Isolation and Identification of Microbes

Enrichment and isolation strategies were implied to isolate the organic acid producing bacteria. To isolate pure cultures, enrichment was carried in 20 mL syringe bottles sparged with N_2 and were allowed for the fermentation up to three days. The samples were checked for the accumulation of organic acids. The samples showing acid accumulation were further diluted up to ten-fold and followed the enrichment cycle. The process was repeated for 4-5 cycles. After 4-5 rounds of enrichment, the cultures were serially diluted up to 10^{-7} and plated in nutrient agar medium. The individual colonies that were streaked in fresh plates and the cultures were subjected for the identification by 16s rDNA sequencing using universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-CACGGATCCTACGGGTACCTTGTTACGACTT-5′) as per the established protocols previously followed in our studies [9]. The sequences were aligned using BioEdit 7.2 version. The obtained sequences were searched in the database to find the similarity using BLASTn and based on the similarity search the isolates were identified.

3. Fermentation with Synthetic Microbial Consortia Using Vegetable Waste

The vegetable slurry was blended with media to the final concentrations as mentioned above and the final media component concentrations were kept constant for all experiments conducted in this study. The fermentation mixture was prepared by mixing vegetable slurry, distilled water (sterile), culture media (sterile), and inoculum. The seed inoculum of the mixed culture was prepared by growing the cultures to OD_{600} 1-1.2 and were pelleted, washed with saline (0.85% NaCl in DW), and resuspended in a sterile culture medium. This was used as the seed inoculum added to the fermentation mix. The pH was adjusted to 6.0, or the desired pH according to the experimental design. Fermentation was conducted in 100 mL serum bottles containing 50 mL of culture medium and sparged with N_2 and sealed. The cultures were grown at 35 °C or temperatures according to the experimental design. The fermentation was conducted at 120 rpm for three days or continued according to the experimental design.

4. Total Organic Acid Quantification

The total organic acid quantification in various experiments was determined by the standard titration method described in the A.O.A.C manual [10] with the optimized protocol in our lab. The brief modified protocol includes the addition of 100 µL phenolphthalein (pH indicator) in 10 mL of DW. The gradient concen-

tration of sodium hydroxide starting from 0.01 M to 1 M sodium hydroxide in 96 well microtiter plate. The dilution point of minimum concentration was detected by pink color change taken as neutralization point and the concentration of acid was calculated as 1 mL of NaOH is equivalent to 90.08 g total organic acid accumulated in the fermentation medium [11]. Hence, taking 90.08 as an equivalent factor, the calculations were made as described in the A.O.A.C manual.

5. HPLC Analysis for the Detection and Identification of Organic Acids Produced in the Medium

High-performance liquid chromatography (HPLC) was used to identify individual organic acids accumulated during fermentation. Based on the RF value of the standards used under Hypersil Gold C18 column (100 mm long 4.6 mm internal diameters, 5 μm) using 50 mM phosphoric acid in acetonitrile as the mobile phase with a flow rate of 0.7 mL/min. The same conditions were used to examine test samples derived from the fermentation medium after passing through 0.22 μm filters (Labware, India). The quantification of each organic acid was determined using a standard curve prepared from standards with known concentrations [3].

6. Quantification of Reducing Sugars

The quantification of reducing sugars was done by Dinitro salicylic acid (DNS) method as described by Miller et al. [12]. The brief protocol as follows. The samples were centrifuged at 12,000 g for 15 min and 1 mL of supernatant was taken for the analysis. 3 mL of DNS reagent was added to the 1 mL of sample and the mixture was incubated in the water bath at 95 °C for 10 min or until red color appeared. The tubes were cooled and 1 mL of 1% Rochelle salt (potassium sodium tartrate tetrahydrate) was added. The tubes were left at room temperature for 20 min to build red earthy color. The optical density was determined by a spectrophotometer [DeNovix, Wilmington, USA] at 540 nm. The quantification was determined by taking the standard curve prepared against the known concentrations of reducing sugar (glucose).

7. Determination of Chemical Oxygen Demand

The chemical oxygen demand (COD) was determined as described by Ma et al. [13]. The brief protocol includes the oxidation of the samples by the closed reflux procedure, and the amount of $K_2Cr_2O_7$ utilized in the oxidized sample was determined by measuring the absorbance of the formed Cr^{3+}. The calculation was made by the amount of used $K_2Cr_2O_7$ proportional to the oxidizable organic matter present in the sample.

8. Optimization Model Development by Response Surface Method

For the determination of the optimum conditions to produce a high titer of organic acids during the fermentation, the central composite design (CCD) model was adopted. The optimization used in this model was based on response surface methodology [14]. Three components, pH, temperature, and inoculum load, were taken as variables [Table 3(b)]. To predict the optimized conditions, Design expert 7.0 (State Ease, Inc., Minneapolis, MN, USA) was used to develop the model. The least-squares technique was applied to decide the polynomial estimation. A focal composite plan was utilized to fit this model. In the CCD, for each coded factor low pivotal, high hub, factorial and an essential issue were coded as −2, −1, 0, +1, and +2, respectively. The examination was done from low to high pivotal. The predicted optimized productivities are shown in the Table 3(a) and 3(b).

9. Analysis of Gas Mixtures

All samples were analyzed for the biogenic gas produced for every 48 h of the interval between each analysis. The gas pressure in the serum bottles was released before the analysis to check the amount of gas accumulated during the fermentation. The gas generated was measured *via* water displacement using water-filled U-shaped reverse graduated measuring tube connected to an airtight septum with a gas needle. The total gas generated in mL was noted. From the serum bottle, 2 μL of gas was collected and injected manually using a gas-tight syringe into the GC (gas chromatography) system to analyze the biogenic gas accumulated during fermentation. Analysis of gas mixture using GC fitted with TCD (thermal conductivity detector). Packed column CARBOXEN 1000 (HP 6890 GC system, USA) was used for gas separation and ultra-pure helium gas was used as the carrier gas. All optimizations and standard curves for the calculation of percentage gas in the mixture were made using standard gas mixtures of N_2, CO_2 and CH_4.

RESULTS AND DISCUSSION

Waste management is a rising issue globally and the traditional undefined landfilling method is becoming a critical issue for environmental safety. Moreover, the cost input is becoming a burden for nations. Vegetable food waste occupies a large portion of the municipal solid waste derived through the consolidated activity of various food supply chains and domestic kitchen activity. Most of the vegetable food waste is managed by landfilling and is becoming a major issue to be considered. Landfilling is responsible for a significant portion of global methane emission; a potent GHG holds a 23-fold higher impact than CO_2 [15,16]. Microbial fermentation is widely accepted for the pre-treatment of wet waste like vegetable food waste. This predetermined microbial process can make vegetable food waste into useful products through the carbon-neutral method. The microbial fermentation process may solve the issue of the environment; however, the economic burden will be a factor of concern for low and middle-income nations [17]. Thus, the present investigation was started with the primary objective of making vegetable waste management from an economic burden to revenue gain.

The hypothesis included in the present study will provide a proof of concept for green disposal of vegetable food waste with neutral carbon emissions as well as a factor of revenue generation. The devised microbial cell factory can produce organic acids and/or fuel from vegetable waste [1]. According to our scheme, as detailed in Fig. 1, by utilizing microbial fermentation, energy-rich vegetable food waste could be used as a carbon and nitrogen source for growing the microbial cell mass and to accumulate industrially valuable chemicals and/or fuels. This process could act as economic reimbursement to the local governance by vegetable waste management. In addition to chemical feedstock (organic acids), the process could also be adapted to generate green energy (CH_4/H_2). To make this process successful at the lab scale, our initial efforts were made to isolate the efficient microbes that can utilize the vegetable food waste to conduct fermentation and produce organic

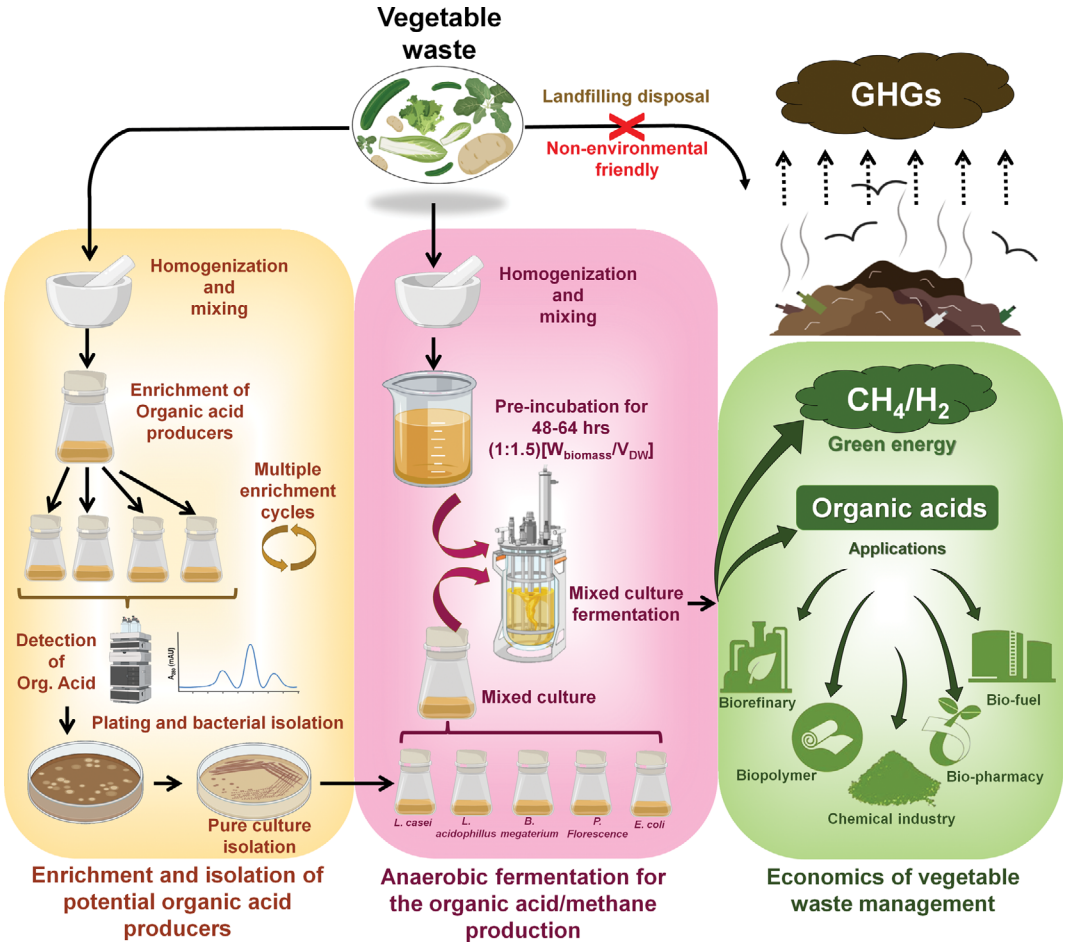

Fig. 1. Schematic presentation of vegetable waste management proposed in this study to produce green chemicals (organic acids) and fuels (CH_4/H_2).

acids or fuel.

1. Isolation of the Microbial Species with the Ability to Ferment Vegetable Food Waste

Several bacterial species were isolated till date which are capable of producing organic acids utilizing various soluble sugars and other fermentable raw materials [1]. However, the cultural and food habits of the different provenances will have a significant role in the composition of vegetable waste generated. Hence, we considered that isolating the microorganisms from waste biomass generated local regions will have better efficiency in utilizing the

Table 1. The isolated bacterial species with the ability to accumulate organic acids from vegetable waste

S. no.	Bacterial species	Organic acid produced	Amount of organic acid detected [pure cultures]
1	*Lactobacillus casei*	Lactic acid	1.23±0.38 g L^{-1}
		Acetic acid	2.36±0.32 g L^{-1}
		Propionic acid	0.59±0.17 g L^{-1}
2	*Lactobacillus acidophilus*	Lactic acid	1.67±0.19 g L^{-1}
		Acetic acid	2.98±0.18 g L^{-1}
		Butyric acid	0.38±0.12 g L^{-1}
3	*Bacillus megaterium*	Acetic acid	2.16±0.37 g L^{-1}
4	*Pseudomonas florescence*	Oxalic acid	3.36±0. 61 g L^{-1}
		Succinic acid	0.62±0.13 g L^{-1}
5	*Escherichia coli*	Acetic acid	1.38±0.16 g L^{-1}
		Formic acid	0.96±0.27 g L^{-1}

waste biomass as a nutrient substrate for organic acid production. Thus, to isolate efficient microbes, the enrichment technique was utilized. This enrichment process was conducted in a minimal buffer medium with 25% (W/V) of 24 h incubated vegetable waste. The samples were incubated at 37 °C in an orbital shaker and were checked for organic acids in regular intervals. Those samples showing the accumulation of organic acids (lactic acid, acetic acid, butyric acid, etc.) were used to isolation of the dominant species responsible for acid production. Five different bacterial species were isolated from the enriched cultures and found to be *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus megaterium*, *Pseudomonas florescence* and *Escherichia coli* [Table 1].

2. Production of Organic Acids with Pure Culture

Each species was checked for the fermentation ability of vegetable waste for the corresponding acid production. Initial experiments were conducted with the sterile medium containing autoclaved vegetable waste mixture homogenized with equal volume of DW (W/V). The mixture was taken up to 10% of the vegetable waste in the medium. The fermentation was conducted for 72 h, and every 24 h of the interval samples were collected for organic acid detection and quantification. The results showed no significant production of organic acids and further bacterial growth was estimated *via* colony-forming units (CFU). Since the medium showed turbidity due to vegetable substrate added to the medium, this did not allow us to estimate the bacterial growth by OD_{600}; hence, we followed the CFU analysis for bacterial growth estimation. The CFU analysis showed that there was only a minor increase in the CFU count in the cultures. This phenomenon may be because the pure cultures used in the study could not utilize the complex carbohydrates and nutrients from the vegetable slurry provided as a substrate [18]. On the other hand, we observed the production of organic acids during the fermentation of vegetable waste during the enrichment steps. This indicates that the natural consortium indigenously colonized in the waste might be assisting in converting complex carbohydrates and other macronutrients into simple sugars and readily consumable nutrients. These nutrients are used by dominant species enriched during fermentation. Further, the acid-producing bacterial species used these simple sugars and other nutrients to accumulate the corresponding organic acids.

Hence, the experiment was conducted with vegetable waste after homogenizing with an equal volume of sterile DW, and allowing it to stand untouched for 72 h. For every 24 h of the interval, the accumulation of reduced sugars was studied. The results showed an increase in concentration of reducing sugars up to 48 h and later reduced. The reduction in the concentration of reducing sugars may be due to the utilization of these sugars by other bacteria in the consortia. Keeping this uninterrupted will result in disproportionate microbial growth, and later, the methanogens scavenge on organic nutrients released during putrefaction and responsible for the methane emissions [19]. This is followed during the natural process in landfilling disposal and responsible for GHGs emissions [20]. We assumed that the vegetable slurry upon incubation for 64 h untouched would be a good substrate for culturing selected bacterial species isolated in this study for organic acid production. Hence, here we used incubated (64 h) slurry for the fermentation studies using isolated microbial species (*Lactobacillus casei*, *Lactobacillus acidophilus*, *Bacillus megaterium*, *Pseudomonas fluorescence,* and *Escherichia coli*) and presumed that the selected species will utilize these simple sugars derived from vegetables/food waste to produce organic acids. Indeed, the experimental results showed that the organic acids were accumulated in the medium, and maximum accumulation was observed up on three days of fermentation and no further increase was observed [Table 1]. Further, the accumulation of reducing sugars in the culture medium was estimated for every 10 h interval and data showed that the sugars accumulation was increased up to 40 h and later reduced. The minimum concentration of reducing sugars in the fermentation medium was observed at 80 h [data was not shown]. In this phase, the polysaccharides were converted to reducing di/monosaccharides by bacterial fermentation. The decline in sugar concentrations at this state may be because the microbes utilized these sugars and converted them into organic acids. There was no increase in the organic acid accumulation after three days of fermentation since they were no accumulation of simple sugars in the medium.

The fermentation results showed that each species could accumulate more than one organic acid during fermentation [Table 1]. Upon 72 h of fermentation, the highest amount of acid accumulated was oxalic acid (3.36±0.61 g L^{-1}) and produced by *P. fluorescence*. The next highest amount of acid accumulated was acetic acid by *L. acidophilus* (2.98±0.18 g L^{-1}), *L. casei* (2.36±0.32 g L^{-1}), followed by *B. megaterium* (2.16±0.37 g L^{-1}), and the least amount was produced by *E. coli* (1.38±0.16 g L^{-1}). In addition, very low quantities of succinic acid and formic acids were also accumulated by *P. fluorescence* and *E. coli*, respectively. This concludes the pre-treatment of the homogenized vegetable waste with indigenous microbial flora acquired during anthropogenic activity through various packaging and distribution chains assisted in the accumulation of soluble sugars. The water activity has a synergetic role in converting complex organic nutrients to simple nutrients. These accumulated simple nutrients were readily utilized by selected species for fermentation. Hence, another experiment was conducted to see the effect of water content during homogenization and use that for the fermentation study. The gradient concentration (1 : 2, 1 : 1.5, 1 : 1.25, 1 : 1, 1 : 0.5, 1 : 0.25, 1 : 0.1 and 1 : 0 [W/V]) of DW was used for homogenization and kept ideal for 80 h. The amount of reducing sugars was estimated for every 10 h of interval. The results showed that 1 : 1.5, *i.e.*, for each 1 g of vegetable waste mixed with 1.5 mL of DW accumulated the highest amount of reducing sugars (1.51±0.289) [Table S1]. Hence, 1 : 1.5 combination of vegetable waste to water for homogenization was maintained for pretreatment for all further experiments in this study.

3. Optimization of Organic Acid Production with Pure Cultures

The technique of pre-treating vegetable slurry with natural flora helped in converting the complex organic components in vegetable waste to a simple readily consumable substrate for the bacterial species. The production concentrations of organic acids accumulated during fermentation were far less than the reported results [21]. Hence, efforts were put forward for the optimization of culture conditions to enhance productivity. The optimization carried out varied with the species under study. Various conditions like media components, salts, pH and temperatures were consid-

ered for the optimization. The optimization results did not show very promising and the overall productivity did not rise more than 15-20%, and in some cases no significant change was observed [Data not shown]. The accumulation of organic acids during the fermentation may depend on various conditions under which the fermentation was carried; among these, the carbon source is one of the major factors that may affect the final productivity [19]. However, our previous experiments showed an adequate amount of soluble sugars getting accumulated in the medium between 48 and 64 h. One more crucial factor that could be considered for the present study was the adaptation of a mixed culture strategy. The cooperative synergy by a group of microbes can play a crucial role in the accumulation of high titer of organic acids from soluble sugars intern released from complex organic waste in our case vegetable waste. Therefore, we believed that using a mixture of bacteria may enhance productivity [22]. In mixed culture fermentation, multiple organisms cooperated during fermentation and significantly enhanced product accumulation and final titer [7,21,23,24]. This was true with our previous studies; it was successfully demonstrated that by utilizing the selective adopted mixed culture could accumulate methane and other organic acids from hydrocarbon-contaminated and coal mining contaminated soils [unpublished data]. Hence, to achieve better productivity and accumulation of organic acids in higher quantity, we attempted to create a synthetic microbial consortium by mixing five bacterial species isolated in this study [Table 1].

4. Organic Acid Accumulation During Fermentation in Vegetable Waste by Synthetic Microbial Consortia

The bacterial cultures were grown to the log phase (1.2 OD_{600}) to make the synthetic consortia for efficient anaerobic digestion and better productivity. The grown cultures after centrifugation, were washed with saline water (0.85% NaCl) and suspended in minimal culture media. The inoculum mix was generated by adding equal quantity of each species (1×10^9 CFU) used as the seed culture. The characteristics of the inoculum and substrate were evaluated for various physicochemical characteristics and the analysis showed that both substrate and inoculum showed ideal characteristics for anaerobic fermentation [Table 2]. The BOD (46,864 mg L^{-1}) and COD (112,000 mg L^{-1}) values of the vegetable biomass used in this study showed rich in organic matter and were considered to be good for acid fermentation. Moreover, the moisture, total solids and suspended solid values were also found to be very much suitable for acid fermentation. The production of various organic acids was evaluated with different amounts of waste

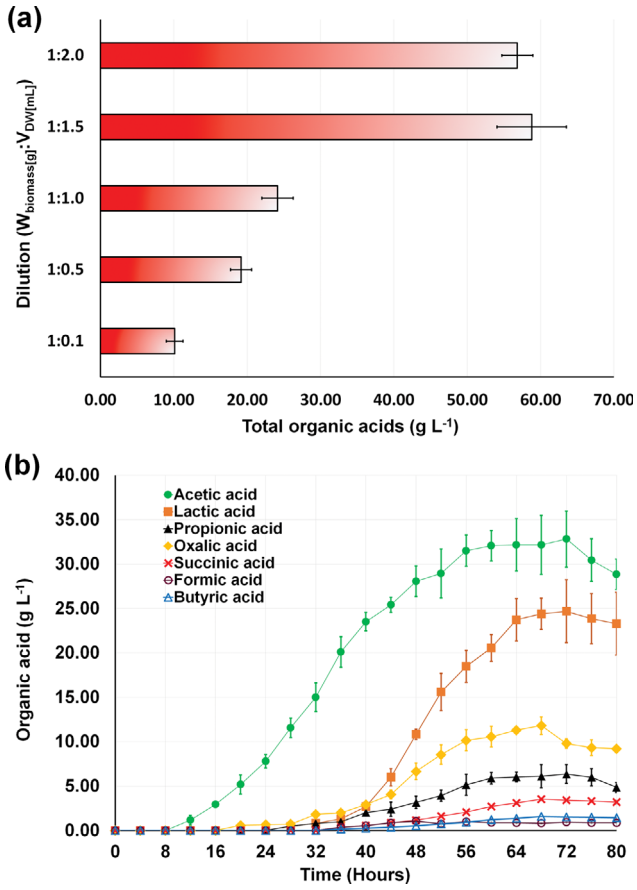

Fig. 2. Organic acids accumulation during acid fermentation by mixed cultures (a) Total organic acid accumulated in various dilutions of biomass with distilled water (W/V); (b) The course of accumulation of individual organic acids during fermentation under optimized conditions [Acetic acid (green), Lactic acid (orange), Oxalic acid (yellow), Propionic acid (black), Succinic acid (Red), Butyric acid (Blue) and Formic acid (brown)].

slurry in liquid media and keeping the bacterial load constant in each experiment. Various concentrations of slurry to media (W/V) [1 : 0.1, 1 : 0.5, 1 : 11 : 1.5, 1 : 2] were taken to evaluate the total organic acid production, results are shown in Fig. 2(a). The best results were found with the ratio of 1 : 1.5 and 1 : 2 vegetable biomass to the culture media. The total organic acid formed in these conditions was observed to be 58.79±4.73 g L^{-1} and 56.83±2.12 g L^{-1},

Table 2. Physicochemical characteristics of substrate and inoculum used in the study

Parameter	Unit	Substrate	Inoculum
pH	-	6.7	6.8
Total solid (TS)	mg L^{-1}	10,673	11,732
Total suspended solid (TSS)	mg L^{-1}	87,600	93,370
Chemical oxygen demand (COD)	mg L^{-1}	112,000	113,000
Biological oxygen demand (BOD)	mg L^{-1}	46,864	48,240
Moisture	%	52.5-57.6	62.9-65.4
CFU	mg L^{-1}	3.3×10^6	1×10^9

Table 3(a). Central composite design (CCD) for optimization of fermentation conditions to produce organic acids from vegetable waste taking three variables each at five levels

Standard order	pH		Temperature (°C)		Inoculum load (1×10^9 cfu/mL)		Total organic acids (g L^{-1})	
	(A)	Coded A	(B)	Coded B	(C)	Coded C	Predicted value	Actual value g L^{-1}
1	4	−2	25	−2	8	−1	33.94	34.32±1.88
2	6	1	35	0	8	−1	42.93	45.13±2.22
3	4	−2	25	−2	8	−1	44.76	40.14±2.38
4	4	−2	35	0	8	−1	37.70	54.50±1.46
5	6	0	25	−2	12	0	50.15	62.31±3.15
6	4	−1	25	−2	12	0	59.92	60.91±2.41
7	6	0	40	1	12	0	65.54	56.13±3.17
8	5	−1	40	1	4	−2	54.32	29.27±1.44
9	4	−2	35	0	20	2	32.21	30.80±0.60
10	8	2	35	0	20	2	33.32	30.16±1.16
11	6	0	30	−2	20	2	26.67	21.55±0.68
12	6	0	35	0	16	1	31.12	37.44±3.27
13	6	0	45	2	16	1	32.14	41.38±1.11
14	6	0	40	1	16	1	68.04	72.44±3.43
15	6	0	30	−1	20	2	70.34	64.70±2.96
16	6	0	30	−1	12	0	68.04	68.71±0.73
17	6	0	35	0	12	0	68.04	67.43±2.40
18	6	0	30	−1	12	0	68.04	61.67±0.41
19	6	0	30	−1	12	0	68.04	66.29±2.94
20	6	0	35	0	12	0	68.04	66.76±2.53

Table 3(b). Level of independent variables taken in the experimental design in CCD model

Variable	Coded level				
	−2	−1	0	+1	+2
pH	4	5	6	7	8
Temperature (°C)	25	30	35	40	45
Inoculum size (mL)	4	8	12	16	20

Table 4. Composition of individual acids identified in the final fermentation medium under optimized conditions

Organic acids	Concentration (g L^{-1})
Acetic acid	25.27±1.26
Lactic acid	19.11±1.73
Propionic acid	6.30±0.82
Oxalic acid	9.43±1.90
Succinic acid	2.78±0.49
Butyric acid	1.20±0.15
Formic acid	0.96±0.15
Total organic acids	65.06±7.27

respectively. Further quantification of individual acid components was determined by HPLC and acetic acid was found to be the dominant acid and accumulated up to 25.27±1.26 g L^{-1} followed by lactic acid (19.11±1.73 g L^{-1}), and the next dominant acid was oxalic acid (8.43±1.90 g L^{-1}). Along with the above acids, propionic acid and succinic acids also accumulated as minor products. Formic acid was a minimal component accumulated during the fermentation [Table 3]. For further improvement in the accumulation, optimization design was conducted according to the central composite design as described in the materials section. For the experimental optimization, the components considered in this model were pH, temperature, and inoculum size. These were taken as representations of the response surface for the suitable combination prediction, and experiments were conducted accordingly [Table 3(a) and (b)]. The total organic acids accumulated in the experiments were found to be near corresponding to the predicted values [Table 3(a)]. The best combination of fermentation conditions with a high quantity of total organic acids accumulated was at 40 °C in pH 6 with 16 mL (1×10^9 CFU mL^{-1}) inoculum load [Table 3(a)]. Up to 72.44±3.43 g L^{-1} of total organic acids were estimated to be produced under these optimized conditions.

Furthermore, the accumulation of different acids during this optimized condition was determined by analyzing the total organic acids by HPLC, and sampling was done every four hours of interval up to 80 h. The results showed that the optimum quantities of organic acid accumulation were observed starting from 60 h and continued until 72 h. Later, the concentrations declined slightly [Fig. 2(b)]. The highest concentration of each organic acid was found to be at 72 h except for oxalic acid and succinic acid. The maximum concentration of these acids was achieved at 64-68 hours and the concentration was observed to be 11.81±0.99 g L^{-1} and 3.53±0.11 g L^{-1}, respectively [Table 3].

The changes in the physicochemical characteristics are well corresponded with the accumulation of organic acids during fermen-

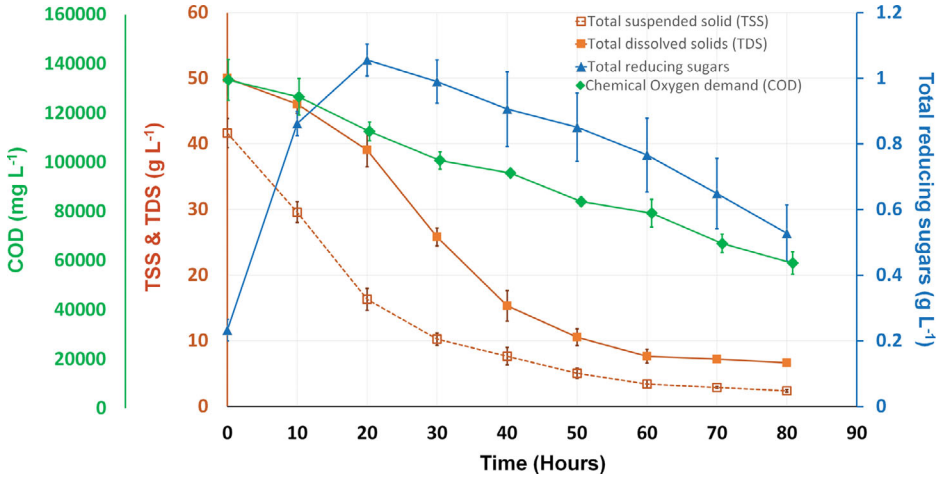

Fig. 3. The course of change in the physicochemical characteristics during the fermentation of vegetable waste biomass for the accumulation of organic acids [COD (Green), Total suspended solid (Brown-dotted line) Total dissolved solids (Brown-solid line) and Total reducing sugars (Blue)].

tation [Fig. 3]. A steep increase in the concentration of total reducing sugars was observed at 20 h and later declined slowly. This might be because the cells started utilizing them for organic acid accumulation. And accordingly, a pH change in the medium was observed [Table S2, Supplementary information]. Unlike pure culture fermentation, mixed culture shows the co-operative microbial metabolism which can assist in optimum production of the product (in our case organic acids). More often, the researchers achieve this by enrichment of the mixed culture by 3-4 cycles of enrichment. However, the enriched cultures are very dynamic and often lose synergy and fail to accumulate target product after a few rounds of fermentation/subculture [25]. Application of known species of microbes to make the synthetic consortia can bypass the steps of enrichment, and once they are well-established, one can recreate the system infinitely. In the present system, the population demonstrated the co-operative metabolic reinforcement via metabolite exchange leading to functional metabolic flux towards the product accumulation. This includes a complex microbial ecological interaction. On the whole, the present established microbial consortia created with the addition of precultures made a synergy with the pre-treated biomass helped in the enhancement of the organic acids titer [26]. Nevertheless, this synergy includes many complex molecular and cellular co-operations and they help in attaining optimum productivity. Furthermore, the knowledge gaps in these aspects are to be filled [22]. The present system developed in our studies can help in understanding these aspects of synthetic consortia, and our future studies may help in filling the knowledge gaps in this area.

When the experiments were continued more than three days, a decrease in organic acids after 72 h was observed. This may be due to the activity of the acid utilizing bacteria, probably the methanogens to generate methane. Hence, further studies were conducted to detect methane production, if any, when the fermentation was continued further.

5. Production of Green Fuels (CH_4/H_2) from Vegetable Waste

Ever since human civilization started, fuel has been the factor of social/national development. A nation's economy in the present context is majorly influenced by the energy resources and technology of efficient energy utilization. Renewable energy technologies sooner are predicted to rule the nation's future in the upcoming time, since fossil fuel reserves are getting depleted. Moreover, the environment is deteriorating upon the overutilization of fossil-based fuels. Biofuels and bioenergy are perhaps the next-generation fuels that will dictate future human evolution. Hence, we made efforts to utilize vegetable waste biomass to produce green fuels. Our preliminary experiments were conducted to check the ability of these microbial consortia to produce methane. Hence, a parallel study on the productivity of methane during the fermentation in a laboratory-scale of 100 mL serum bottles was conducted. The procedures were followed as per the optimized conditions derived in the above sections; however, incubation was extended to 22 days. The gases accumulated in the bottles during the anaerobic fermentation were analyzed. The results showed that there was an accumulation of methane during the anaerobic digestion by the microbial consortia, and the methane generation was started from

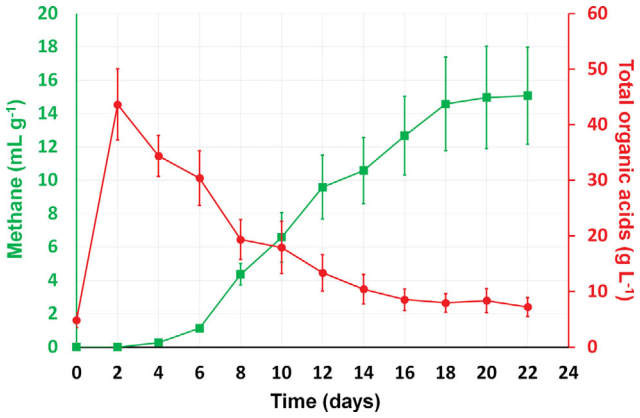

Fig. 4. The course change in organic acid accumulation and methane production during anaerobic digestion of vegetable waste [Methane (Green) and Total organic acid (Red)].

day four and continued up to 17 days, and there was no further increase in the production [Fig. 4].

During fermentation, the organic acids accumulated till 72 h and started to decline up to 22 days. Productivity was determined to be 14.97 mL g^{-1} biomass. Interestingly, in the initial days of fermentation, a trace amount of hydrogen was detected, and this could be the result of fermentation by *E. coli via* formate hydrogen lyase (FHL) system [1]. Under the anaerobic conditions, *E. coli* generates one molecule of acetate and one molecule of formate from pyruvate, formate is used by the FHL system to generate H_2 and CO_2. The disappearance of the H_2 in the later stages may be due to the following reasons: (i) the H_2 generated may be utilized by the cell for reducing the NAD^+, (ii) the H_2 can also participate in the reversible reaction by hydrogenase to produce H^+, (iii) the most probable reason may be the methanogens, where slow-growing *Archaea* consume the H_2 for the metabolism and utilize the organic acids for the production of methane. In conclusion, the methane accumulation was initiated from day four and continued till the organic acid was depleted in the medium, and no soluble sugars were able to generate from the biomass [Fig. 4]. Although the methane production was detected in the anaerobic digestion, the productivity of the methane in the present investigation was very low compared with the other established anaerobic processes using various waste biomass [27-29]. The final titer and yields of H_2/CH_4 in this present demonstrated process were not good enough to develop a profitable industrial application. However, the experiments in this study give proof of concept to generate clean energy [CH_4/H_2] from the vegetable waste biomass along with valuable organic acids. Two prospects could be drawn from this study: one, by exploring the modern biotechnology/molecular biology techniques the metabolic flux engineering of *E. coli* could result in the production of pure clean energy (H_2), which does not elicit any carbon footprint upon combustion. The second option is, the addition of suitable methanogen enriched consortia to the fermentation system established in the present study could result in a high accumulation of methane. This can have an industrial success with the economic benefit of waste management. Either way, the process will become a good example of waste management by converting vegetable waste into green fuels. However, to establish a profitable system, more systematic experimentations are required to make the system carbon neutral and economically potential. Nevertheless, the overall process developed in this study will have two benefits: (i) utilization of vegetable food waste as feedstock for microbial fermentation will result in the production of valuable organic acids with minimal cost, and (ii) it will create a platform for facile and green waste management practice and help to eliminate indirect carbon emissions.

CONCLUSION

Organic acids are the widely used organic building blocks in the synthesis of many valuable chemicals and act as a precursor for producing fuels. This study demonstrated that the conversion of the waste biomass (vegetable food waste) into organic acids could be used as feedstock for various green syntheses of industrially important chemicals. The results achieved show the importance of native bacterial species and comprehending the advantage of creating synthetic microbial consortia for the efficient accumulation of various organic acids. This study also proved the advantage of utilizing the natural microbial flora acquired during the various stages of the food processing chain to help in the accumulation of simple sugars which could be later utilized by the selective microbial consortia for the accumulation of organic acids. This study is the first to demonstrate a unique system for the green waste management of vegetable waste. The present study demonstrates the production of organic acids to the optimum level and is the only report by utilizing vegetable food waste. This system develops a novel technique of applying synthetic microbial consortia made by selective indigenously isolated bacterial species. This study also provides proof of concept for the possible production of green fuels from vegetable waste; however, further studies are much needed for the establishment of efficient and economically profitable technology. The elucidated system in this study could comprehensively answer two primary issues: (i) the economic burden incurred in vegetable waste management, and (ii) the carbon footprint being added to the globe by traditional landfilling waste disposal.

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SUPPORTING INFORMATION

Additional information as noted in the text. This information is available via the Internet at http://www.springer.com/chemistry/journal/11814.

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